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Genetic Basis of Antifungal Drug Resistance

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Abstract

Antifungal resistance caused by mutations of the drug target, overexpression of the drug target, and drug efflux by the upregulation of transporters is increasingly common. Recently our understanding of fungal drug resistance has been advanced by the identification of three key transcriptional regulators of resistance: Tac1p, Upc2p, and Mrr1p. The discovery of hyperactive variants of these regulators in resistant clinical isolates confirms the importance of transcriptional regulation in the development of antifungal resistance. Alternative mechanisms of drug resistance including aneuploidy and biofilm formation have recently been documented in fungi; as well as the phenomenon of drug tolerance. Characterization of the transcriptional regulation of fungal drug resistance and the identification of novel mechanisms of resistance has implications for current therapy and for the development of future antifungal drugs.

I. Introduction

Fungal infections have increased in frequency over the past two decades as a result of the rising numbers of immunocompromised patients. *Candida*, *Cryptococcus* and *Aspergillus* remain the major human fungal pathogens but previously rare fungal pathogens such as the zygomycetes and hyaline molds are emerging, driven by the use of antifungals to which these species are intrinsically resistant. Intrinsic and acquired antifungal resistance is a growing clinical problem for all fungal pathogens. However, most research and drug development remains focused on *Candida* species. Consequently this review focuses on antifungal resistance mechanisms that have been identified in the major fungal pathogens, with a particular emphasis on *Candida*. While *Candida* research findings often have implications for other fungal pathogens, it will be vital to expand basic research, diagnostics and drug development to encompass the expanding spectrum of human fungal pathogens. Here we summarize preceding work on well described resistance mechanisms [as previously reviewed in 1› 2] in the context of recent novel discoveries.

II. Antifungal drugs and their targets

The existing antifungal armamentarium contains four classes of drug- polyenes, nucleic acid synthesis inhibitors, ergosterol biosynthesis inhibitors and echinocandins. Clinical resistance has been observed for all classes of antifungal and no single class of antifungal is effective against all invasive mycoses. Each class of drug has a specific mode of action and a distinct role in the treatment of fungal pathogens.

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A. Polyenes

The polyenes are the oldest class of antifungal and still have an important clinical role due to their broad spectrum and the rarity of resistance. Polyenes target the end product of ergosterol biosynthesis by binding directly to ergosterol to intercalate into fungal cell membranes creating pores. The use of amphotericin B deoxycholate, the most common polyene, is limited by acute infusion-related reactions and substantial nephrotoxicity. Newer lipid formulations are less nephrotoxic and have a broad antifungal spectrum [1, 2].

Due to this broad spectrum the primary use of polyenes is for empiric treatment. Polyenes are effective against *Candida* species, *Cryptococcus* and other dimorphic fungi such as *Histoplasma, Blastomyces, Coccidioides*, and *Paracoccidiodomyces*. Polyenes are also active against more resistant, emerging yeasts species such as *Rhodotorula, Geotrichum, Trichosporon* and zygomycetes. Polyenes are fungicidal against most yeasts and molds [1, 2].

B. Nucleic acid synthesis inhibitors

5-flucytosine (5FC) is the main antifungal drug that targets nucleic acid synthesis. 5FC is imported into fungal cells and converted to the metabolically active nucleoside analog 5-fluorouracil which inhibits DNA replication. 5FC is used exclusively in combination with polyenes or azoles due to the rapid development of resistance when it is used as a single agent [1, 2].

C. Ergosterol Biosynthesis Inhibitors

Ergosterol biosynthesis inhibitors (EBIs) include the azoles, morpholines, thiocarbamate and allylamines. These drugs all work by inhibiting the biosynthesis of ergosterol but each has a distinct target enzyme. Azoles work by inhibiting the fungal lanosterol 14α-demethylase, a cytochrome p450 enzyme that is required for ergosterol biosynthesis, commonly denoted Erg11p. Inhibition of Erg11p depletes cellular ergosterol and causes the accumulation of toxic sterol intermediates. The azoles are generally fungistatic against yeast but some triazoles have fungicidal activity against certain molds [1, 2].

The azoles are the most important and commonly used EBI and have been in use for more than two decades and include the imidazoles and the triazoles. Imidazoles (ketoconazole, miconazole and clotrimazole) are currently used for the treatment of superficial fungal infections and have limited use for treating invasive mycoses.

The first-generation triazoles including fluconazole and itraconazole are still used to treat a range of superficial and invasive fungal infections. Fluconazole has good overall activity against *Candida* species and *Cryptococcus neoformans*. However, some non-*albicans Candida* species such as *C. krusei* and some isolates of *Candida glabrata* display decreased susceptibility to fluconazole [1].

The second-generation triazoles such as voriconazole and posaconazole have improved broad-spectrum activity. Voriconazole is effective against most fungi including *C. neoformans, Aspergillus, Scedosporium* and *Fusarium* species [1]. Posaconazole, licensed in 2007, is the newest second-generation triazoles. It also has the broadest spectrum of activity of the azoles and is the only azole drug with activity against zygomycete fungi [3].

D. Echinocandins

The echinocandins are the most recent class of antifungal drug. Echinocandins work by inhibiting (1, 3)-β-D-glucan synthase. Glucan is a key component of the fungal cell wall and inhibition of glucan synthase disrupts fungal cell wall integrity. Echinocandins are fungicidal

against most yeasts, while fungistatic against most molds [1, 3]. Echinocandins have no activity against cryptococcal species and non-*Aspergillus* molds, although the cryptococcal (1,3)-β-D-glucan synthase is inhibited by echinocandins *in vitro* [4].

III. Mechanisms of Antifungal Drug Resistance

Mechanisms of antifungal drug resistance are heterogeneous with respect to level of resistance, stability, and fitness cost. Here we discuss resistance caused by alterations in drug targets by mutation, alterations in transcriptional regulation, reduced drug accumulation due to efflux, the genetic basis of drug tolerance, and several alternative mechanisms.

A. Resistance genes

Mutations that lead to resistant alleles of drug targets are discussed below according to drug class.

i. Azoles -Point mutations in Erg11p—Point mutations that result in amino acid substitutions in lanosterol demethylase are a well characterized mechanism of azole resistance. To be effective such mutations must decrease the affinity of the enzyme for drug without impairing its cellular function. These constraints generally confine amino acid substitutions to particular hot spots in the target enzyme. Specific point mutations in the around the active site of *Candida* Erg11p render the enzyme resistant to inhibition by some triazoles [5]. Similar point mutations have been identified in Erg11p homologs of *A. fumigatus* [6] and of *C. neoformans* [7].

ii. Echinocandins-Point mutations in Fks1p—Several mutations in the glucan synthase gene *FKS1* are sufficient to drastically reduce susceptibility to echinocandins in yeasts and molds [14]. Mutations in glucan synthase arising during echinocandin therapy have been documented in several *Candida* species [15-17]. A single amino acid change in Fks1p of *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* seems to account for the intrinsic reduced susceptibility to echinocandins of these species [18].

iii. 5FC -Inactivation of import—Mutation of the genes necessary for 5-FC toxicity has been studied in detail in *Candida lusitaniae*, especially as these mutations can cause cross resistance to fluconazole [19, 20]. 5FC is imported by a cytosine permease (Fcy2p); it is then deaminated by a cytosine deaminase (Fcy1p) to 5FU. 5FU is converted to 5-fluorouridine monophosphate by a phosphoribosyltransferase (Fur1p). Inactivation of any of these genes confers resistance to 5FC. *FCY1* and *FCY2* mutants also display fluconazole resistance in the presence of subinhibitory 5FC concentrations but not to fluconazole alone. Two hypotheses have been proposed to explain this cross resistance. The first is that extracellular 5FC acts as a competitive inhibitor of FLC uptake [20] however this does not explain cross-resistance mediated by Fcy1p. More recently it has been proposed that cross resistance is mediated by the accumulation of fluorinated cytosine within the cell by an unknown mechanism. 5FC enters cells with mutant *FCY2* permeases through other lower affinity permeases [19], indicating that *FCY2* mutation does not abolish 5FC import.

B. Transcriptional regulation of drug resistance

i) Upc2p hyperactivity—Resistant clinical isolates are often found to overexpress *ERG11* and other ergosterol biosynthetic genes [8, 9]. Upc2p is the major regulator of ergosterol biosynthesis and has been found to upregulate ergosterol biosynthesis in response to azole drugs [10]. Recently, azole inducibility through Upc2p was found to depend on an azole-responsive enhancer (ARE) element in the promoter of *ERG11* and other genes in the ergosterol biosynthetic pathway [11]. The ARE is an imperfect inverted repeat recognized by Upc2p and

is necessary and sufficient for Upc2p azole induction. The mechanism by which azoles induce gene expression is Upc2p-dependent but the azole activation mechanism of Upc2p is unknown. We have hypothesized that Upc2p is activated by directly sensing decreased sterol levels in the fungal cell membrane [10, 12].

A recent report identified a gain of function mutation in Upc2p as a mechanism of azole resistance [13], (Table 1.1). The mutation was identified in a clinical isolate of *C. albicans* and analysis of a matched susceptible isolate revealed that this mutation was acquired during azole therapy. The mutation in Upc2p causes constitutive overexpression of *ERG11* and other *ERG* genes. The mutation also decreased the susceptibility to terbinafine, another EBI. It is not yet known how this mutation impacts resistance to other antifungals nor has the mechanism of constitutive activation of this mutation been determined.

ii. Efflux Pumps—Two classes of drug efflux pumps, the ABC transporters (Cdr1p and Cdr2p in *Candida*) and the major facilitator transporters (Mdr1p in *Candida*) have been characterized in fungi. Cdr1p homologs have been characterized across *Candida* species and in *C. neoformans* [21]. So far, the ABC transporters *Afu*Mdr1p, *Afu*Mdr2p, *Afu*Mdr4p, and AtrFp, and the major facilitator, *Afu*Mdr3p are believed to mediate azole efflux in *Aspergillus* [22].

Fungal efflux pumps mediate drug resistance through inducible (drug-responsive) pathways and constitutive (continual overexpression) pathways. Constitutive overexpression of multidrug efflux pumps is a major cause of clinical resistance to fluconazole [1, 2] Echinocandins are not believed to be substrates for fungal efflux pumps but it has been reported that overexpression of *CDR2* can result in reduced susceptibility to some echinocandins by agar dilution assay [23].

a) Inducible Expression of Fungal Efflux: The *Candida* transcription factors Tac1p, Fcr3p and Ndt80p are thought to be positive regulators of inducible *CDR1* and *CDR2* expression in *Candida* [1, 2]. Tac1p is the best characterized of these regulators and has been found to regulate *CDR1* and *CDR2* transcription by binding to a conserved drug-responsive element (DRE) in their promoters [24] (Table 1.1). The transcription factors Mrr1p, Cap1p and Mcm1p have been shown to regulate inducible expression of the major facilitator Mdr1p in *Candida* through three distinct promoter binding sites [25].

Nuclear-receptor mediated induction of ABC transporters: A novel mechanism for induction of fungal efflux with striking functional similarities to vertebrate pathways was recently described in the fungal pathogen *C. glabrata* [26]. *C. glabrata* is an emerging fungal pathogen with intrinsic azole resistance thought to be due to reduced azole accumulation [27]. Pdr1p is the major transcriptional regulator of ABC transporters in *C. glabrata.* Pdr1p was shown to bind directly to structurally dissimilar drugs and then bind a subunit of the mediator complex, Gal11p. Binding of Gal11p by Pdr1p induces expression of ABC transporters. This mechanism of induction is conserved in *Saccharomyces cerevisiae* but it is not known if it exists in other fungal species. It is possible that this pathway plays a role in the increased intrinsic azoles resistance of *C. glabrata* and *S. cerevisiae* relative to *C. albicans*. If the pathway is conserved between fungal species it may provide a novel target for co-therapies that specifically inhibit fungal efflux.

mRNA stability of CDR1: Post-transcriptional regulation of mRNA stability has recently been identified as a mechanism of efflux pump overexpression [28]. A recent report describes increased stability of *CDR1* mRNA and greater rates of transcriptional initiation for *CDR1* in the azole resistant isolate of a matched pair of susceptible and resistant isolates. The authors

hypothesize that differences in the 3′ UTR of *CDR1* are responsible for decreased message turnover leading to overexpression of Cdr1p.

b) Constitutive overexpression of Efflux Pumps: Constitutive overexpression of multidrug efflux pumps is an important and clinically relevant cause of resistance. Two regulators of inducible expression of efflux pumps Tac1p and Mrr1p mediate constitutive overexpression due to gain of function mutations [29, 30].

Tac1p hyperactivity: As discussed above, Tac1p mediates inducible and constitutive upregulation of the *CDR1* and *CDR2* genes. Hyperactive alleles of *TAC1* cause high level azole resistance by constitutive upregulation of *CDR1* and *CDR2*. Two mutations in the putative Tac1p transactivation domain have been identified that cause hyperactivity (Table 1.1) [24, 29]. Hyperactive Tac1p is recessive as azole resistance is only observed in strains homozygous for the mutation [29]. Loss of heterozygosity at the *TAC1* locus to unmask the recessive allele can occur by mitotic recombination or by chromosomal duplication.

Mrr1p hyperactivity: Like Tac1p, the zinc cluster transcription factor Mrr1p controls inducible and constitutive Mdr1p expression [30]. Hyperactive Mrr1p alleles have been identified in resistant clinical isolates, and many different amino acid substitutions cause Mrr1p hyperactivity [31] (Table 1.1).

C. Alternative mechanisms of Drug Resistance

i) Overexpression of Pdr16p by Tac1p—The Tac1p regulon has recently been shown to include the *C. albicans* gene *PDR16* [32-34]. *PDR16* encodes a putative phosphatidylinositol transfer protein thought to be important for phospholipid homeostasis. Overexpression of *PDR16* increases azole resistance twofold, while deletion of *PDR16* increases azole susceptibility [32]. *PDR16* is co-induced with the multidrug transporter genes *CDR1* and *CDR2*, which also function as general phospholipid translocators [35]. It has been proposed that Tac1p regulates the asymmetric distribution of phospholipids in the lipid bilayers of membranes and that this influences azole susceptibility [33]. This hypothesis is supported by the recent identification of *CB4* (putative sphingosine kinase), *RTA3* (putative phospholipid flippase), and orf 19.1887 (putative lipase), as putative Tac1p targets by genome wide expression profiling [34].

ii) Aneuploidy—Aneuploidy is a way of increasing gene copy number by whole chromosome duplication or other genomic rearrangements. Genome alterations are induced as an adaptive response when cells are stressed. Aneuploidy is a recently recognized mechanism of target overexpression and efflux pump overexpression in *Candida* and has found to be prevalent in clinical azole resistant isolates [37]. A particular isochromosome consisting of two left arms of chromosome 5 fused head to head is frequently associated with azole resistance [38, 39]. This resistance is thought to be due to gene amplification of *ERG11* (encoding the azole target) and *TAC 1* (encoding a positive regulator of efflux pump genes) both of which are found on the left arm of chromosome 5. Aneuploidy has been also been identified as a mechanism of azole resistance in an isolate of *C. glabrata* that duplicated the entire chromosome encoding the *ERG11* homolog *CYP51* [40].

iii. Biofilms—Biofilms are surfaced-attached microbial communities embedded in an extracellular matrix. Many *Aspergillus*, *Cryptococcus* and *Candida* bloodstream and urinary tract infections are associated with biofilms on indwelling medical devices. Fungal biofilms are refractory to most antifungals and these infections pose major treatment challenges. Fungi that are genotypically drug-susceptible become highly resistant to most antifungals when cultivated as biofilms but resistance is not retained if cells are switched to planktonic growth.

Many mechanisms contribute to the resistant phenotype of fungal biofilms including: upregulation of efflux pumps, decreased drug diffusion, decreased growth rate and oxidative stress resistance [41, 42]. A recent report identifies the novel of contribution of the extracellular matrix to the drug resistance of biofilms [43]. The authors proposed that β-1, 3 glucans in the matrix bind and sequester fluconazole from cells. β-1, 3 glucans were found to bind fluconazole directly. Glucanase increased the susceptibility of biofilms to fluconazole but had no effect on the fluconazole susceptibility of planktonic cells, presumably because glucanase degrades β-1, 3 glucans of the extracellular matrix allowing azoles to reach cells.

D. Drug tolerance

Drug tolerance is the phenomenon of reversible adaptive resistance that occurs by modification of gene expression. A series of recent studies have identified the heat shock protein Hsp90p and one of its client proteins calcineurin as key modulators of the fungal stress response [44]. Both Hsp90 and calcineurin mediate fungal stress responses which alter global gene regulation resulting in drug tolerance. Stress responses act as a buffer that protects cells from antifungal toxicity and allows the selection of stable antifungal resistant mutants.

i) Calcineurin and Hsp90—Calcineurin is a highly conserved serine-threonine-specific protein phosphatase. Calcineurin is an essential component of the fungal stress response. Genetic mutation or chemical inhibition of the calcineurin pathway renders fungi extremely susceptible to a variety of cellular stresses, including drug treatment. Calcineurin inhibitors make fungistatic agents such as azoles fungicidal in *Candida*. The combination of calcineurin inhibitors and azoles are also effective against azole resistant *Candida* isolates, as well as biofilms. A positive interaction between calcineurin inhibitors and echinocandins is also seen for *Aspergillus* [see ref 44 for a complete review].

ii) Increased chitin synthesis rescues cells from echinocandins—Some isolates of *C. albicans* survive and grow in high concentrations of caspofungin, a phenomenon known as paradoxical growth or Eagling [45]. A recent study demonstrates that the mechanism of paradoxical growth is increased chitin synthesis mediated by the stress response pathways [46]. Echinocandins cause cell wall damage which triggers the stress response signaling pathways mediated by Protein Kinase C (PKC), High-Osmolarity Glycerol (HOG) and calcineurin. Activation of these cellular stress responses trigger cell wall salvage pathways, which upregulate chitin synthesis allowing cells to grow in otherwise lethal concentrations of drug. The authors found that paradoxical growth is blocked by chitin synthase inhibitors which are synergistic with echinocandins.

IV. The impact of antifungal drug resistance on fitness and virulence

The development of resistance in fungi is relatively slow compared to bacteria due to the lack of horizontal gene transfer, limited patient to patient transmission and longer generation times. However, experiments in the development of fungal drug resistance suggest that contrary to bacteria, once stable antifungal resistance is acquired it is maintained even in the absence of drug [47]. Thus the paradigm of resistance mutations incurring fitness and virulence costs is not always borne out in fungi. In fact, constitutive overexpression of ABC transporters and several other resistance genes are accompanied by a gain in fitness both in the presence and in the absence of the drug *in vitro* [48].

A recent study examined the virulence of two drug resistant strains of *C. albicans*: a fluconazole-resistant isolate overexpressing *MDR1* and a caspofungin resistant isolate with a homozygous mutation in the *FKS1* gene [49]. The resistant isolates were significantly more pathogenic than the susceptible parent strain in a systemic mouse model in the absence of drug. The increased systemic virulence of the drug-resistant strains was attributed to differences in

cell wall composition, increased filamentation, increased adherence, and enhanced biofilm formation. It is important to note that both resistant isolates were generated by *in vitro* drug exposure, thus it is unclear if this observation has clinical relevancy.

Conversely, azole resistance mediated by inactivation of *ERG3* has been shown to negatively impact virulence. Mutation of *ERG3* allows cells to bypass the production of toxic sterols in the presence of azoles. *ERG3* bypass mutants are rare among clinical isolates and it is uncertain if this mutation confers azole resistance *in vivo* [50].

A. Intrinsic Drug Resistance

Antifungal prophylaxis has created a niche in immunocompromised patients that favors the emergence of intrinsically drug resistant strains of fungi. Previously rare species are becoming more common including non-*albicans Candida*, non-*fumigatus Aspergillus*, opportunistic yeast-like fungi such as *Trichosporon* and *Rhodotorula*, zygomycetes and hyaline molds like *Fusarium* and *Scedosporium* [51]. The emergence of intrinsically resistant species correlates with the introduction and widespread use of antifungal drugs and necessitates novel drugs that are effective against a broader spectrum of fungal species.

B. Unidentified resistance mechanisms

The optimization of current antifungal therapy and the development of novel therapeutics are aided by the continued characterization of antifungal resistance mechanisms. Many antifungal resistance mechanisms remain to be identified as evidenced by collections of resistant clinical isolates in which no known mechanisms of resistance have been identified [9]. Many common drug resistance mechanisms found in bacteria have not been identified in fungi. We speculate here on two possible resistance mechanisms which have not been validated experimentally.

It is likely that other mechanisms of efflux exist in *Candida* and other fungal species, based on the presence of many predicted transporters in fungal genomes. We have screened a collection of resistant *Candida* isolates for efflux of the fluorescent xenobiotic Rhodamine 6G (R6G). R6G has antifungal activity and high levels of R6G efflux were observed in resistant clinical isolates that do not overexpress *CDR1*, *CDR2* or *MDR1* (our unpublished observation). It is unknown if the increased efflux we observed was due to R6G induction of known efflux pumps or by constitutive efflux by an unknown mechanism.

Azoles must enter fungal cells to exert an effect. There is evidence that azoles enter fungal cells by facilitated diffusion [52] but a specific transporter has not been discovered. Mutations in an azole transporter could exclude azole drugs from a cell conferring high level resistance. It is also possible that intrinsic resistance of some fungal species is due to lower affinity azole transporters or other mechanisms of azole exclusion as has been suggested for *C. glabrata* [40].

Degradation of drug by cellular enzymes is a well characterized resistance mechanism in bacteria. It is possible that this mechanism also exists in the fungi. Fungi are known to secrete aspartyl proteases (SAPs) that are important for nutrient acquisition and virulence. We have determined that the echinocandin caspofungin is not degraded by SAPs (data not shown). However, SAPs or other fungal proteins may destroy or sequester antifungal drugs outside of the cell.

V. Conclusion

Antifungal resistance is often a factor in life-threatening fungal infections. The complexity of antifungal resistance is evidenced by the diverse mechanisms discussed here. Analysis of sequential clinical isolates from patients undergoing antifungal therapy shows that resistance

mechanisms rarely exist in isolation. Multifactorial resistance evolves overtime as fungi are exposed to drugs [1, 2]. The characterization of resistance mechanisms in isolates from treatment failures remains a powerful guide for understanding clinically significant resistant mechanisms.

Just as there are striking similarities in resistance mechanisms between different fungal species, there are common pathways that may be useful drug targets. Efflux mediates drug resistance in diverse fungal organisms. Drugs that interfere with efflux could render previously resistant strains newly susceptible to current therapy. Stress response signaling pathways are also well conserved and calcineurin has already proven a highly effective target for combination therapy *in vitro* in several fungal species [53]. Inhibiting calcineurin may have the additional benefit of decreasing the ability of fungi to survive in the stressful environment of the human host [54]. In light of the resistance mechanisms discussed here and the emergence of novel fungal species, development of novel antifungals to old and new targets will be required to keep pace with the remarkable evolution of fungal drug resistance.

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Table 1

Transcriptional regulators of antifungal resistance.

Transcription factors	Target genes	Binding site	Hyperactive alleles	Reference
Upc2p	*ERG* genes *CDR1*, *MDR1*, *YOR1*	ARE: TCGTATA(13)-AATATCG	G648D	[18*,54**]
Tac1p	*CDR1*, *CDR2* *PDR16*	DRE: CGG(4)CGG	N977D, N972D, G980E, A736V, T225A	[29*,33]
Mrr1p	*MDR1*	-	K335N, Q350L, T360I, T381I, P683S, N803D, P683H, R873T, G878E, A880E W893R, T896I, G997V,L998F	[30**,31*,55*]